

Evidence That Receptor Activator of Nuclear Factor (NF)- κ B Ligand Can Suppress Cell Proliferation and Induce Apoptosis through Activation of a NF- κ B-independent and TRAF6-dependent Mechanism*

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The receptor activator of NF- κ B ligand (RANKL), a recently identified member of the tumor necrosis factor (TNF) superfamily, has been shown to induce osteoclastogenesis and dendritic cell survival. Most members of the TNF superfamily suppress cell proliferation and induce apoptosis, but whether RANKL does so is not known. We demonstrate that treatment of monocyte RAW 264.7 cells with RANKL induces dose-dependent growth inhibition (IC_{50} = 10 ng/ml) as determined by dye uptake and [3 H]thymidine incorporation methods. Suppression of RANKL-induced NF- κ B activation by dominant-negative I κ B α or by the NEMO-peptide had no effect on RANKL-induced cell growth inhibition. Inhibition of RANKL-induced JNK activation, however, abolished the RANKL-induced apoptosis. Suppression of interaction of RANK with TRAF6 by TRAF6-binding peptide abrogated the anti-proliferative effects of RANKL, suggesting the critical role of TRAF6. Flow cytometric analysis of cells treated with RANKL showed accumulation of cells in G₀/G₁ phase of the cell cycle, and this accumulation correlated with a decline in the levels of cyclin D1, cyclin D3, and cyclin E and an increase in cyclin-dependent kinase inhibitor p27 (Kip). Flow cytometric analysis showed the presence of annexin V-positive cells in cultures treated with RANKL. RANKL-induced apoptosis was further confirmed using calcein AM/ethidium homodimer-1 dye and cleavage of poly(ADP-ribose) polymerase (PARP), procaspase 3, and procaspase 9; benzoyloxycarbonyl-VAD, the pancaspase inhibitor, suppressed the PARP cleavage. Thus, overall, our studies indicate that RANKL can inhibit cell proliferation and induce apoptosis through a TRAF6-dependent but NF- κ B-independent mechanism.

Human receptor activator of NF- κ B ligand (RANKL)¹ (TNFSF11/TRANSGEN/OPGL/ODF), a cytokine independently dis-

covered by four different groups (1–4), is a type II transmembrane protein of 317-amino acid length. It belongs to the TNF superfamily and has ~30% homology to the TNF-related apoptosis-inducing ligand (TRAIL) and to CD40L and about 20% homology to Fas ligand. It exists in two forms: a 40- to 45-kDa cellular form and a 31-kDa soluble form derived by cleavage of the full-length form at positions 140 or 145 (4). RANKL mRNA is expressed at the highest levels in bone and bone marrow and in lymphoid tissues (lymph node, thymus, spleen, fetal liver, and Peyer's patches) (1–4). Its major role in bone is the stimulation of osteoclast differentiation (4, 5) and inhibition of osteoclast apoptosis (6). In addition, RANKL has a number of effects on immune cells, including activation of c-Jun N-terminal kinase (JNK) in T cells (1), inhibition of apoptosis of dendritic cells (7), induction of cluster formation by dendritic cells, and alterations in cytokine-activated T cell proliferation (2). RANKL knockout mice have severe osteopetrosis with defects in tooth eruption (8). They also have a complete absence of osteoclasts. In addition, they exhibit defects in early differentiation of T and B cells, lack lymph nodes, and have defects in thymic differentiation and mammary gland development (8, 9).

RANKL mediates its effects by specifically binding to receptor activator of NF- κ B (RANK) (10), a member of the TNF receptor superfamily. RANK is a 616-amino acid long receptor with a 383-amino acid intracellular domain that lacks any significant homology to other members of this family (2). Although RANK is ubiquitously expressed in human tissues, its cell surface expression is limited to dendritic cells, the CD4+ T cell line MP-1, foreskin fibroblasts, osteoclast precursors, and certain Hodgkin lymphomas (2, 10, 11). RANK knockout mice lack peripheral lymph nodes and have defective T and B cell maturation but do not have any defect in thymic development (12).

RANK activation by RANKL is followed by its interaction with TNF receptor-associated factors (TRAF) family members TRAF2, TRAF3, TRAF5, and TRAF6 (13–16), which in turn recruits NF- κ B-inducing kinase, which itself leads to activation of NF- κ B-inducing kinase and activation of NF- κ B (15). RANKL has also been shown to activate c-Fos, JNK, c-Src, and

* This work was supported by the Clayton Foundation for Research (to B. B. A.), Department of Defense, U. S. Army Breast Cancer Research Program Grant BC010610 (to B. B. A.), PO1 Grant CA91844 on lung chemoprevention from the National Institutes of Health (to B. B. A.), and a P50 Head and Neck Specialized Programs of Research Excellence grant from the National Institutes of Health (to B. B. A.). The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "advertisement" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

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factor- κ B ligand; RANK, receptor activator of NF- κ B; NF- κ B, nuclear factor- κ B; TNF, tumor necrosis factor; TRAF, TNF receptor-associated factor; IKK, I κ B kinase; TRAP, tartrate resistance acid phosphatase; I κ B α -DN, I κ B α dominant-negative I κ B α ; EMSA, electrophoretic mobility gel shift assay; JNK, c-Jun N-terminal kinase; PI, propidium iodide; PARP, polyadenosine-5'-diphosphate-ribose polymerase; HRP, horseradish peroxidase; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-2H-tetrazolium bromide; z-VAD, benzoyloxycarbonyl; FITC, fluorescein isothiocyanate; PBS, phosphate-buffered saline; FACS, fluorescence-activated cell sorting; Cdk, Cyclin-dependent kinase.

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serine/threonine kinase Akt/PKB (2, 10) (17). TRAF6 and NF- κ B play an indispensable role in osteoclast differentiation as demonstrated by the osteopetrotic phenotype of TRAF6 and NF- κ B knockouts (18, 19).

The prosurvival role of RANK/RANKL through activation of NF- κ B and subsequent up-regulation of Bcl- x_L has been demonstrated (1, 6). Whether RANKL can also inhibit cell proliferation and induce apoptosis, as other TNF family members do, is not known. To test this hypothesis, we treated macrophage RAW 264.7 cells with RANKL. In the present report, we provide evidence that RANKL can suppress the proliferation of cells leading to apoptosis and that this process is mediated through a TRAF6-dependent but NF- κ B-independent mechanism.

EXPERIMENTAL PROCEDURES

Materials—Rabbit polyclonal antibodies against cyclin D1, cyclin D2, cyclin D3, cyclinE, p27 (Kip), and PARP and an Annexin V kit were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). Antibodies against caspase 3 and caspase 9 were purchased from Cell Signaling Technology (Beverly, MA). TRAP staining was performed using a leukocyte acid phosphatase kit (387-A) from Sigma (St. Louis, MO). Cell-permeable TRAF6 binding peptide with or without a Kaposi fibroblast growth factor leader sequence (AAVALLPAVLLALLAP-RKIPT-EDEYTDPRSPQST; leader sequence in italics), and mutant TRAF6 binding peptide (AAVALLPAVLLALLAP-RKIATADEATDRSPQST; leader sequence in italics and mutated amino acids underlined), NEMO (NF- κ B essential modifier; IKK γ)-binding domain peptide (NBD) with or without an antennapedia homeodomain leader sequence (DRQIKI-WFQNRMRKWKK-TALDWSWLQTE; leader sequence in italics) and leader sequences alone, mouse monoclonal anti-RANK antibody were kind gifts from Imgenex (San Diego, CA). Goat anti-rabbit-HRP conjugate was from Bio-Rad Laboratories (Hercules, CA), and goat anti-mouse-HRP was from BD Transduction Laboratories (Lexington, KY). 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyl-2H-tetrazolium bromide (MTT) and anti- β -actin antibody were from Sigma-Aldrich Chemicals (St. Louis, MO). z-VAD, a pancaspase inhibitor, and SP600125, an antrypyrrolone inhibitor of Jun N-terminal kinase (JNK), were purchased from Calbiochem (San Diego, CA). Dulbecco's modified Eagle's medium/F-12, fetal bovine serum, and 100 \times antibiotic-antimycotic solution were purchased from Invitrogen (Grand Island, NY). [γ - 32 P]ATP was purchased from ICN Pharmaceuticals (Costa Mesa, CA). A live/dead cell death detection kit was from Molecular Probes (Eugene, OR). Recombinant RANKL and RAW264.7 cells stably expressing FLAG-1kBa-DN under tetracycline-repressible promoter were kindly provided by Dr. Bryant G. Darnay (M. D. Anderson Cancer Center, Houston, TX).

Cell Culture—Murine monocytic cell line RAW 264.7 cells were obtained from American Type Culture Collection (Rockville, MD) and were cultured in Dulbecco's modified Eagle's medium/F-12 medium containing 10% fetal bovine serum and a 1 \times antibiotic-antimycotic mixture. Occasionally cells were tested by Hoechst staining and by custom polymerase chain reaction for mycoplasma contamination.

Cell Surface Expression of RANK—For analysis of cell surface expression of RANK, cells were harvested and suspended in Dulbecco's phosphate-buffered saline containing 1% fetal bovine serum and 0.1% sodium azide. The cells were preincubated with 10% goat serum for 20 min and washed, and then monoclonal mouse IgG anti-RANK antibody was added. Following 1-h incubation at 4 $^{\circ}$ C, the cells were washed and incubated for an additional 1 h in a FITC-conjugated goat anti-mouse IgG monoclonal antibodies. The cells were analyzed using a FACSCalibur flow cytometer and CellQuest acquisition and analysis programs (BD Biosciences, San Jose, CA).

MTT Assay—The anti-proliferative effects of RANKL on RAW 264.7 cells were determined by the MTT dye uptake method as described earlier (20). Briefly, the cells (2000/well) were incubated in triplicate in a 96-well plate in the presence or absence of RANKL in a final volume of 0.1 ml for the indicated time periods at 37 $^{\circ}$ C. Thereafter, 0.025 ml of MTT solution (5 mg/ml in PBS) was added to each well. After a 2 h incubation at 37 $^{\circ}$ C, 0.1 ml of the extraction buffer (20% SDS, 50% dimethylformamide) was added, incubation was continued overnight at 37 $^{\circ}$ C, and then the OD was measured at 570 nm using a 96-well Multiscanner Autoreader (Dynatech MR 5000), with the extraction buffer as blank. The anti-proliferative effect was determined by the formula, anti-proliferative effect = $[1 - (\text{OD of the experiment samples}/\text{OD of the control})] \times 100$.

Thymidine Incorporation Assay—The anti-proliferative effects of RANKL were also monitored by the thymidine incorporation method (20). For this, 2000 cells in 100 μ l of medium were cultured in triplicate in 96-well plates in the presence or absence of RANKL. Six hours before the completion of the experiment, cells were pulse-treated with 0.5 μ Ci of [3 H]thymidine, and the uptake of [3 H]thymidine was monitored using a Matrix-9600 β -counter (Packard Instruments, Downers Grove, IL). The anti-proliferative effect was then determined by the formula, anti-proliferative effect = $[1 - (\text{cpm of the experiment samples}/\text{cpm of the control})] \times 100$.

Preparation of Nuclear Extracts for NF- κ B—Nuclear extracts were prepared according to Schreiber *et al.* (21). Briefly, 1×10^6 cells were washed with cold PBS and suspended in 0.4 ml of hypotonic lysis buffer containing protease inhibitors for 30 min. The cells were then lysed with 12.5 μ l of 10% Nonidet P-40. The homogenate was centrifuged, and supernatant containing the cytoplasmic extracts was stored frozen at -80° C. The nuclear pellet was resuspended in 25 μ l of ice-cold nuclear extraction buffer. After 30 min of intermittent mixing, the extract was centrifuged, and supernatants containing nuclear extracts were secured. The protein content was measured by the Bradford method.

Electrophoretic Mobility Shift Assay for NF- κ B—NF- κ B activation was analyzed by electrophoretic mobility gel shift assay (EMSA) as described previously (22). In brief, 8 μ g of nuclear extracts prepared from treated or untreated cells were incubated with 32 P end-labeled 45-mer double-stranded NF- κ B oligonucleotide from human immunodeficiency virus-1 long terminal repeat (5'-TTGTTACAAGGGACTTTC-CGCTGGGGACTTTCCAG GGAGGCGTGG-3') for 15 min at 37 $^{\circ}$ C, and the DNA-protein complex was resolved in a 6.6% native polyacrylamide gel. The radioactive bands from the dried gels were visualized and quantitated by using a PhosphorImager (Amersham Biosciences) using ImageQuant software.

c-Jun Kinase Assay—The c-Jun kinase assay was performed by a modified method as described earlier (14, 15). Briefly, 200 μ g of whole-cell extract was treated with anti-JNK1 antibodies, and the immunocomplexes so formed were precipitated with protein A/G-Sepharose beads (Pierce Chemical). The kinase assay was performed using washed beads as source of enzyme and GST-Jun-(1-79) as substrate (2 μ g/sample) in the presence of 10 μ Ci of [γ - 32 P]ATP per sample. The kinase reaction was carried out by incubating the above mixture at 30 $^{\circ}$ C in the kinase assay buffer for 15 min. The reaction was stopped by adding SDS sample buffer, then boiling. Finally, protein was resolved on a 10% reducing gel. The radioactive bands of the dried gel were visualized and quantitated by using the PhosphorImager as described above.

Western Blot—Whole cell extracts were prepared in the lysis buffer (20 mM Tris, pH 7.4, 250 mM NaCl, 2 mM EDTA, pH 8.0, 0.1% Triton X-100, 0.01 mg/ml aprotinin, 0.005 mg/ml leupeptin, 0.4 mM phenylmethylsulfonyl fluoride, and 4 mM NaVO $_3$). Lysates were then spun at 14,000 rpm for 10 min to remove insoluble material. 30–50 micrograms of extracts were resolved on 10% SDS-PAGE gel. After electrophoresis, the proteins were electrotransferred to a nitrocellulose membrane, blocked with 5% nonfat milk, and probed with antibodies against cyclin D1, cyclin D2, cyclin D3, cyclin E, p27 (Kip) (1:3,000), caspase 3, caspase 9 (1:1,000), or β -actin (1:10,000). Thereafter, the blot was washed, exposed to HRP-conjugated secondary antibodies for 1 h, and finally detected by chemiluminescence (ECL, Amersham Biosciences, Arlington Heights, IL). For detection of PARP, lysates were resolved on a 7.5% polyacrylamide gel and probed with PARP antibodies (1:3000). Anti-PARP antibody detects both intact PARP of 116-kDa and its 85-kDa cleaved fragment.

Live/Dead Assay—Because the presence of growth factors in the serum may antagonize the anti-proliferative and apoptotic effects of RANKL, the cells were serum-starved prior to stimulation with RANKL. Cells were first incubated in absence of serum for 2 days and then treated with 100 ng/ml RANKL for 2 days. Cells were stained with the live/dead assay reagents for 30 min at room temperature. Cell viability were then examined under fluorescence microscope and counted for live/dead (green/red) ratio.

Flow Cytometric Analysis of Cellular DNA—To determine the effect of RANKL on the cell cycle, RAW 264.7 cells were treated for different times, washed, and fixed with 70% ethanol. After incubation overnight at -20° C, cells were washed with PBS, stained with propidium iodide (PI), and then suspended in staining buffer (PI, 10 μ g/ml; Tween 20, 0.5%; RNase, 0.1% in PBS). The cells were analyzed using a FACS Vantage flow cytometer that uses the Cell Quest acquisition and analysis program (BD Biosciences, San Jose, CA). Cells were gated to exclude cell debris, cell doublets, and cell clumps. To check the RANKL-induced cell death, cells were first serum-starved for 2 days prior to RANKL treatment, and the cells were fixed and stained with PI as

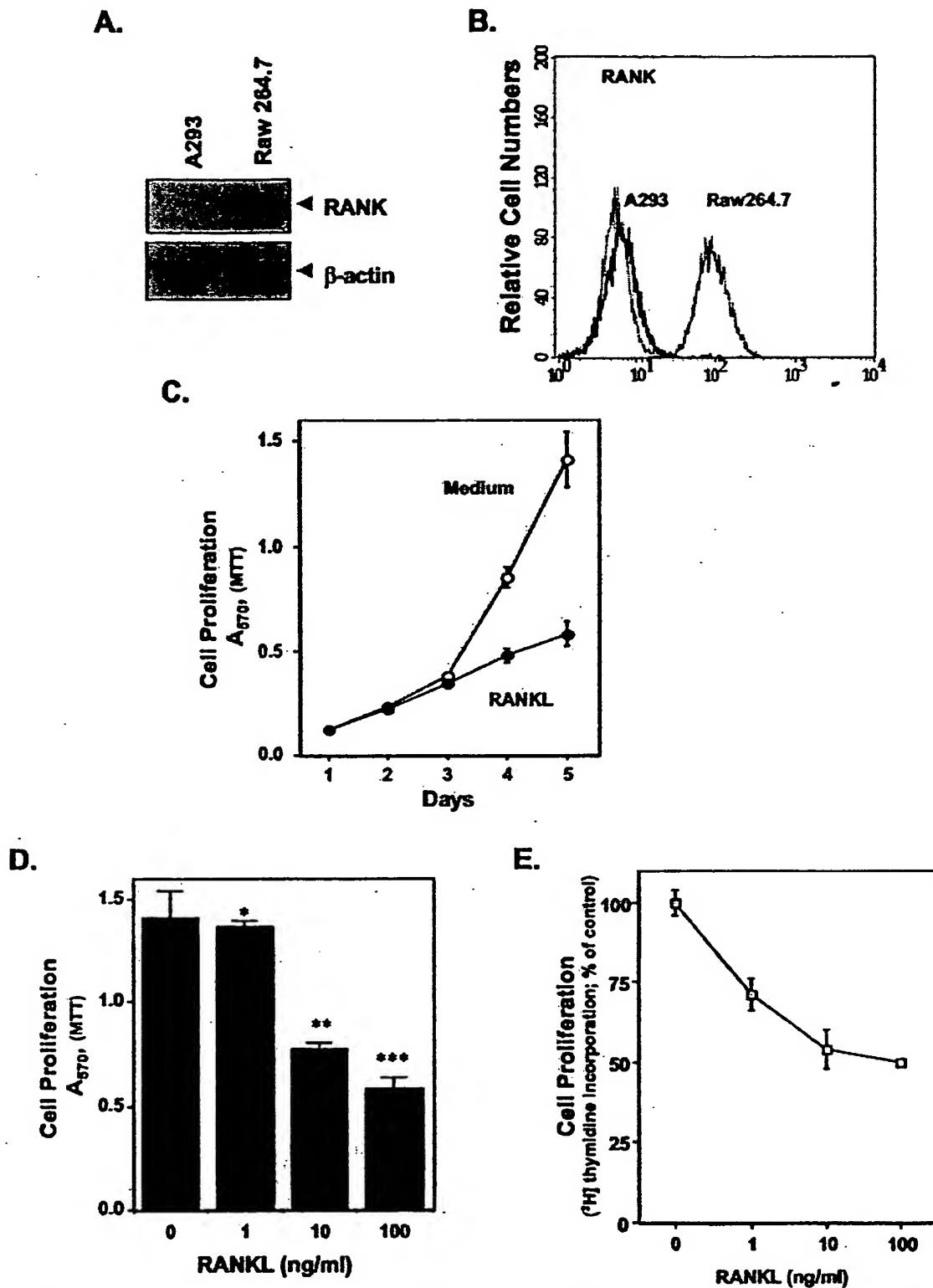


FIG. 1. RANKL inhibits cell proliferation of monocytic RAW 264.7 cells. **A**, RAW 264.7 cells express receptors for RANKL. The whole cell extracts from RAW 264.7 and A293 (negative control) cells were resolved on 7.5% SDS-PAGE and then probed with anti-RANK antibody. **B**, cell surface expression of RANK in RAW 264.7 cells. RAW 264.7 and A293 cells were harvested, labeled with anti-RANK antibody, followed by FITC-conjugated goat anti-mouse IgG antibody. Negative controls (labeled with second-step antibody only) are shown in shaded lines. **C**, RAW 264.7 cells (2×10^5 cells) were incubated either alone or in the presence of RANKL (100 ng/ml) for indicated days and cell growth was measured by the MTT method. For **D** and **E**, cells were incubated with different concentrations of RANKL for 5 days and cell growth was measured by MTT (**D**) or [3 H]thymidine incorporation methods (**E**). Values are means \pm S.D. (indicated as error bars) of triplicate. In **D**, the significance of the results when compared with untreated control is *, $p < 0.001$; **, $p < 0.01$; and ***, $p < 0.005$.

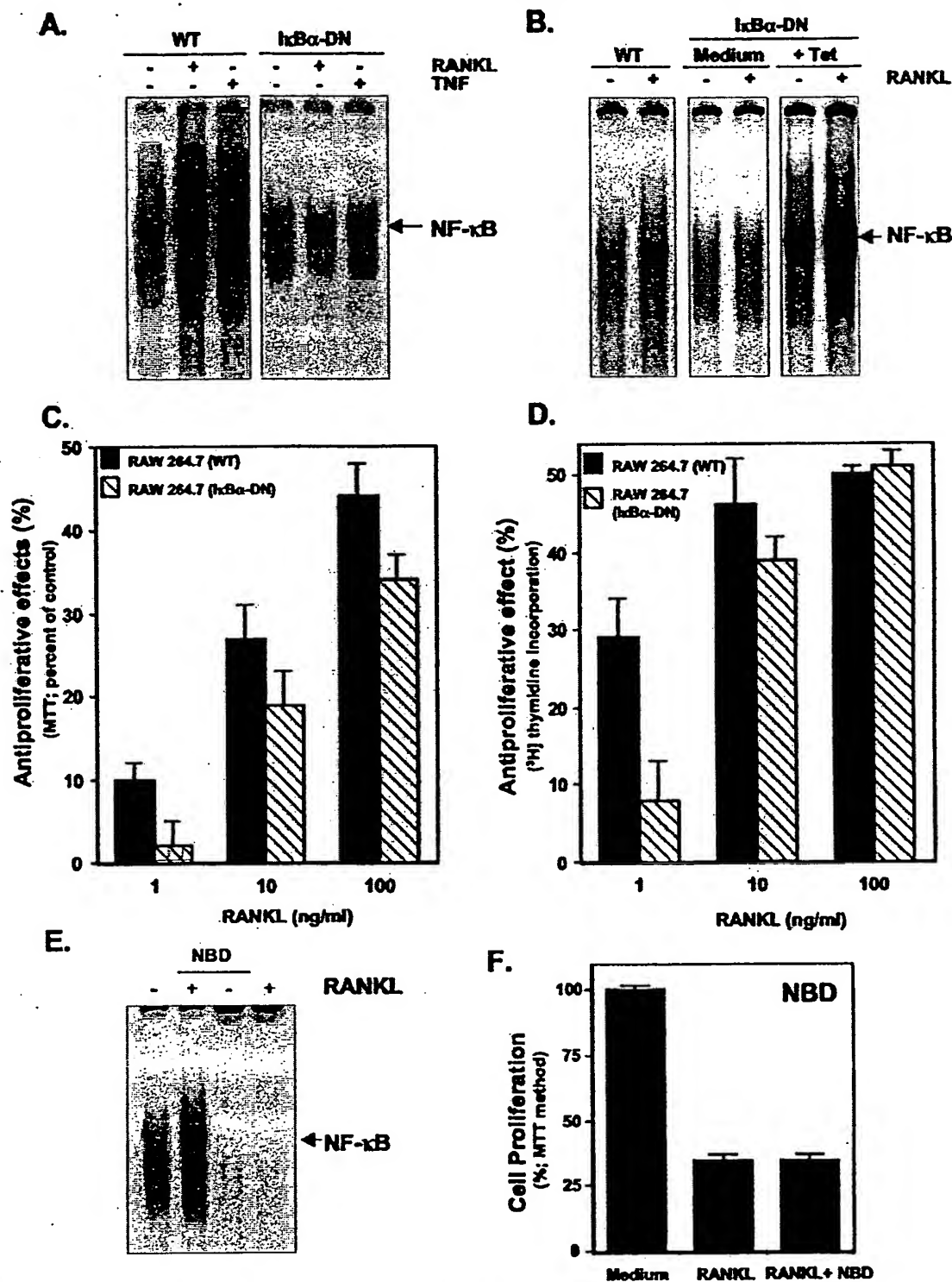


FIG. 2. NF- κ B activation is dispensable in the anti-proliferative effect of RANKL. RAW 264.7 wild type (WT) or I κ B α -dominant negative stably transfected cells (I κ B α -DN) (1×10^6 cells) were treated with RANKL (200 ng/ml) or TNF (1 nM) for 30 min (A) or incubated alone or in the presence of RANKL (200 ng/ml) or TNF (1 nM) for 30 min (B) and tested for nuclear NF- κ B by EMSA as described under "Experimental Procedures." C and D, RAW 264.7 (WT) and RAW264.7 (I κ B α -DN) cells (2×10^5 cells) were incubated either alone or in the presence of different concentrations of RANKL (1, 10, and 100 ng/ml) for 4 days, and cell growth was measured by MTT (C) or [3 H]thymidine incorporation method (D). Inhibition of cell growth was measured as described under "Experimental Procedures." E, RAW 264.7 cells (1×10^6 cells) were incubated alone or in the presence of 100 μ M NEMO-binding domain peptide (NBD) for 2 h. Cells were then treated with RANKL (100 ng/ml) for 30 min, and nuclear extracts were checked for NF- κ B by EMSA. F, RAW 264.7 cells (2×10^5 cells) were preincubated with 100 μ M NBD for 2 h and cultured in the absence or presence of RANKL (100 ng/ml) for 5 days. Cell growth was measured by the MTT method. Values are means \pm S.D. (indicated as error bars) of triplicates.

A.

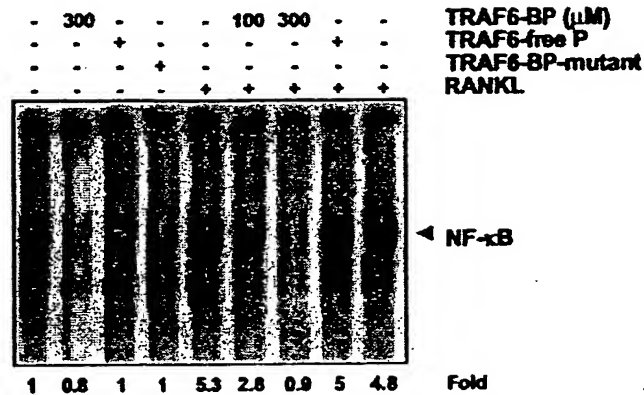
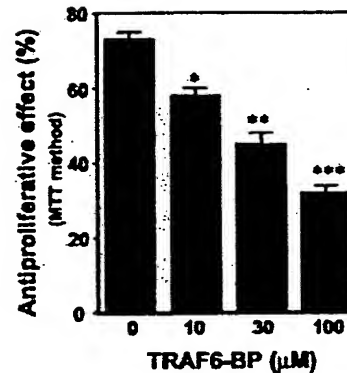


FIG. 3. TRAF6-binding peptide (TRAF6-BP) blocks NF- κ B activation by RANKL and prevents growth inhibitory activity. A, RAW 264.7 cells (1×10^6 cells) were either incubated alone or in the presence of indicated concentrations of TRAF6-BP for 2 h. Cells were then treated with RANKL (200 ng/ml) for 30 min, and nuclear extracts were checked for NF- κ B activation by EMSA. For controls, TRAF6-BP without the delivery peptide (300 μ M; TRAF6-free P), and TRAF6-BP-mutant (300 μ M) were used. B, RAW 264.7 cells (2×10^5 cells) were preincubated with the indicated concentrations of TRAF6-BP for 2 h and then cultured in the absence or presence of RANKL (100 ng/ml) for 5 days, and cell growth was measured by the MTT method. Values are mean \pm S.D. (indicated as error bars) of triplicates. The significance of results when compared with the RANKL-treated control are *, $p < 0.01$; **, $p < 0.005$; and ***, $p < 0.001$.

B.



indicated above. The apoptotic cells with hypodiploid DNA content were detected in the sub-G₁ region in the FL-2A channel.

Annexin V Staining—RANKL-treated cells were washed in PBS and resuspended in 100 μ l of binding buffer containing FITC-conjugated Annexin V. Cells were analyzed by flow cytometry after the addition of PI. Annexin V binds to those cells that express phosphatidylserine on the outer layer of the cell membrane, and PI stains the cellular DNA of cells with a compromised cell membrane. This allows for live cells (unstained with either fluorochrome), which appear in the left lower quadrant, to be discriminated from early apoptotic cells in right lower quadrant (stained only with Annexin V), late apoptotic cells in the right upper quadrant (stained with both Annexin V and PI), and necrotic/dead cells in left upper quadrant (stained only with PI) (23).

RANKL-induced Differentiation of RAW 264.7 Cells—For this RAW264.7 cells were cultured in 24-well dishes at a density of 1×10^4 cells per well and were allowed to adhere overnight. Medium was then replaced, and the cells were treated with 100 ng/ml RANKL. Cultures were stained for TRAP expression as described (24) using an acid phosphatase kit at different days, and photographs were taken using a Nikon Coolpix camera (Nikon, Japan).

Statistical Analysis—All experiments were conducted in triplicate for at least three times. The statistical significance of difference between test groups was analyzed by the Student's *t* test (two-tailed). The difference was considered significant when the *p* value was < 0.05 .

RESULTS

In the present report we investigated whether RANKL, like other members of the TNF superfamily, modulates the proliferation of monocytic RAW 264.7 cells and if so by what mech-

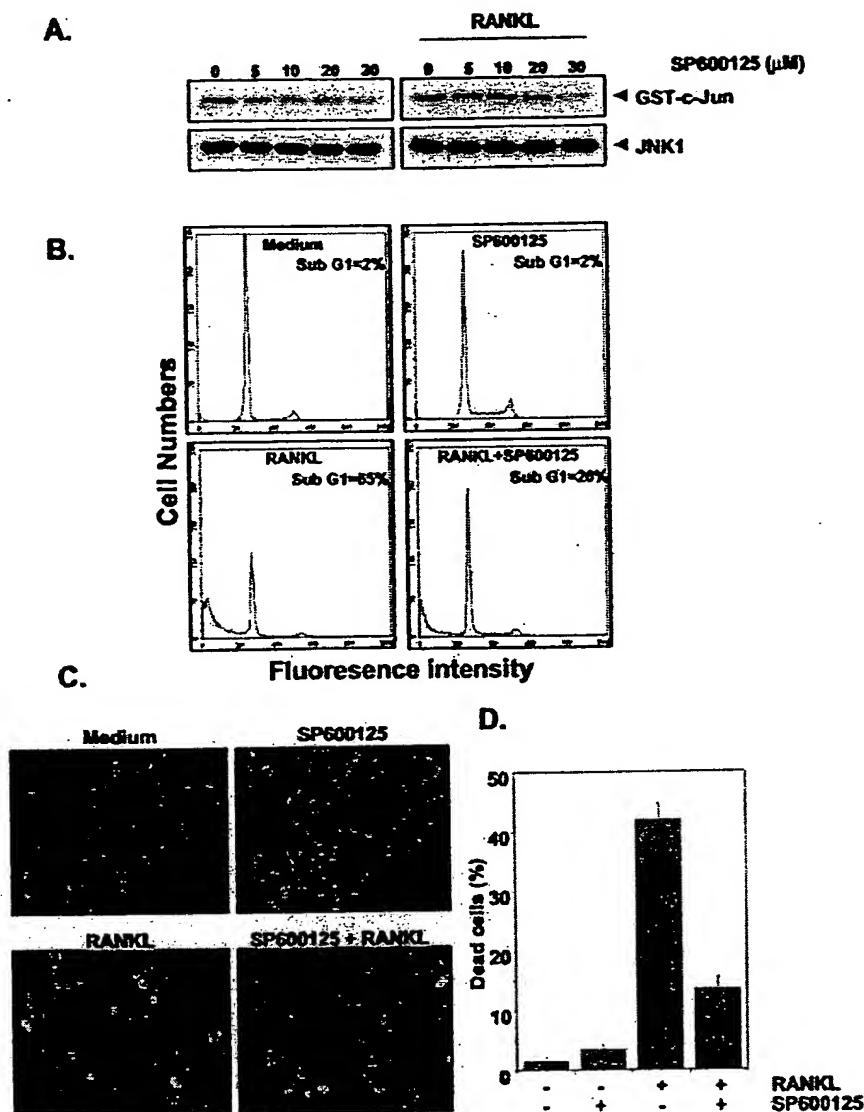
anism. This cell line was found to express RANK on its surface as indicated by Western blot analysis (Fig. 1A) and FACS analysis (Fig. 1B). These results are in agreement with a previous report (25).

RANKL Inhibits the Proliferation of RAW 264.7 Cells—RANKL (100 ng/ml) significantly inhibited the time-dependent proliferation of RAW cells (Fig. 1C). Almost 55% inhibition of cell growth was noted after 5 days of treatment. The growth inhibitory effect of RANKL was dose-dependent (Fig. 1D). Because the MTT method detects cumulative mitochondrial activity in cultures, we confirmed the inhibitory effect of RANKL by the thymidine incorporation method (Fig. 1E).

Growth Inhibitory Effects of RANKL Are NF- κ B Activation-independent—To determine the role of NF- κ B in RANKL-induced growth inhibition, RAW 264.7 cells stably transfected with a dominant negative I κ B α (I κ B α -DN) under the control of a tetracycline-repressible promoter were treated with RANKL and TNF (used as a positive control). Both cytokines activated NF- κ B in WT cells but not in I κ B α -DN-transfected cells (Fig. 2, A and B).

We then treated both WT and I κ B α -DN-transfected RAW264.7 cells with different concentrations of RANKL and measured mitochondrial activity by the MTT method (Fig. 2C) or DNA synthesis by [3 H]thymidine incorporation (Fig. 2D). RANKL inhibited the proliferation of RAW264.7 (I κ B α -DN)

FIG. 4. Suppression of JNK inhibits RANKL-induced anti-proliferative effects. A, RAW 264.7 cells (1×10^6 cells) were incubated with different concentrations of JNK inhibitor (SP600125) for 1 h and then treated with the RANKL (100 ng/ml) for 15 min. Whole cell extracts were prepared, immunoprecipitated with anti-JNK1 antibody, and performed JNK immunocomplex kinase assay using GST-c-Jun as the substrate. B, JNK inhibitor blocks RANKL-induced apoptosis. RAW 264.7 cells (1×10^6 cells) were cultured for 2 days in a serum-free media, then incubated with 10 μ M JNK inhibitor for 1 h, and thereafter treated with RANKL (100 ng/ml) in the presence of serum for 2 days. The cells were washed, fixed, stained with propidium iodide, and analyzed for DNA content by flow cytometry as described under "Experimental Procedures." Histograms indicate total cell population in the cultures. Numbers indicate the percentage of cells with hypodiploid or sub-G₁ DNA content. C, JNK inhibitor blocks RANKL-induced apoptosis. RAW 264.7 cells (2×10^4 cells) were first cultured in the absence of serum for 2 days and then treated with RANKL (100 ng/ml) in the presence of serum for 2 days. Cell death was determined by calcein AM-based live/dead assay as described under "Experimental Procedures." Graphical data of the percentage of dead cells were shown (D). Values are means \pm S.D. (indicated as error bars) of triplicates.



cells almost to the same extent as observed for RAW 264.7 (WT) cells. The RANKL-induced growth inhibition was slightly lower in cells lacking NF- κ B activation than in the control, and this effect was more pronounced at a lower concentration (1 ng/ml) than higher concentration of RANKL (100 ng/ml).

To confirm the lack of a role of NF- κ B in RANKL-induced growth suppression, we used NBD, which blocks the activation of IKK by preventing the interaction of IKK β with its regulatory subunit IKK γ (20). EMSA of nuclear extracts from RAW cells preincubated with NBD for 2 h and then treated with RANKL showed that RANKL-induced NF- κ B activation was inhibited (Fig. 2E). The control peptide, either lacking delivery sequence or lacking NBD, had no effect (data not shown). As shown in Fig. 2F, NBD peptide had no effect on the anti-proliferative effect of RANKL. Thus our results indicate that the anti-proliferative effects of RANKL are independent of NF- κ B activation.

TRAF6-binding Peptide (TRAF6-BP) Reverses the Anti-proliferative Effects of RANKL—A synthetic TRAF6 binding peptide (TRAF6-BP) with a cell permeable Kaposi's fibroblast growth factor leader sequence that can suppress RANKL signaling (24) was used to treat RAW cells before treatment with RANKL. The addition of TRAF6-BP inhibited RANKL-induced

NF- κ B activation (Fig. 3A). The TRAF6-BP, which lacks the delivery sequence or TRAF6-BP-mutant, which has mutation in TRAF6-BP, had no effect (Fig. 3A). As shown in Fig. 3B, TRAF6-BP reversed the growth inhibitory effect on the RANKL in a dose-dependent manner. Thus these results indicate that anti-proliferative effects of RANKL require TRAF6 recruitment.

RANKL-induced JNK Activation Mediates RANKL-induced Cell Death—RANKL is also a potent activator of JNK (14, 15, 17). Whether RANKL-induced JNK activation mediates anti-proliferative effects of RANKL, was investigated. Recently, a specific inhibitor of JNK, which is cell-permeable, selective, and reversible, has been identified (25). SP600125, an anthrapyrazolone, is an ATP-competitive inhibitor of JNK. It exhibits over 300-fold greater selectivity for JNK as compare with ERK1 or p38 MAPK. We used this inhibitor to investigate the role of JNK in RANKL signaling. Results in Fig. 4A show that RANKL activates JNK in RAW cells and the inhibitor SP600125 suppressed JNK activation in a dose-dependent manner. We then examined the effect of this JNK inhibitor on RANKL-induced apoptosis by FACS analysis (Fig. 4B), and by live/dead assay (Fig. 4C). FACS analysis showed that Sub-G₁ fraction of cells remains unchanged on treatment of cells with

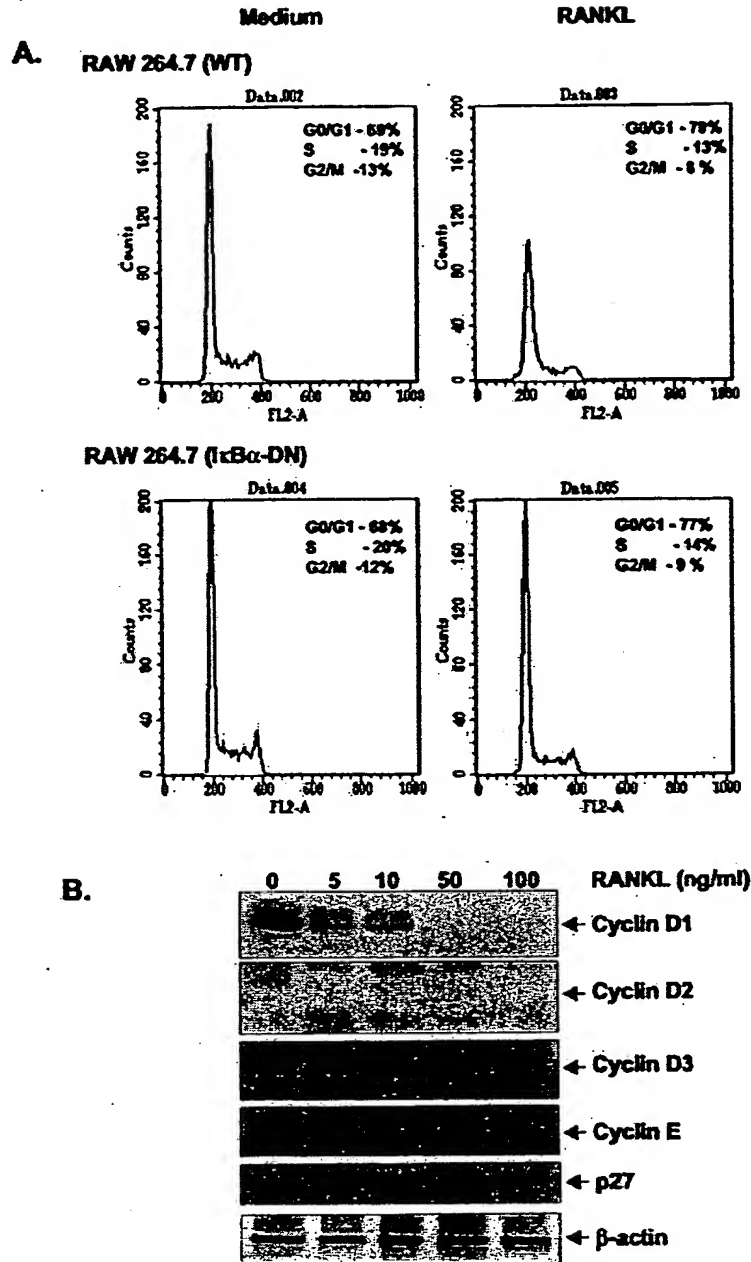


FIG. 5. RANKL blocks the cell cycle at G_0/G_1 phase, inhibits the expression of cyclin D1, cyclin D3, and cyclin E, and induces p27 (kip) expression. **A**, WT and $I\kappa B\alpha$ -DN RAW 264.7 cells (2×10^4 cells) were incubated in the absence or presence of RANKL (100 ng/ml) for 4 days. Thereafter, the cells were washed, fixed, stained with propidium iodide, and analyzed for DNA content by flow cytometry as described under "Experimental Procedures." Histograms indicate single cells undergoing different phases of the cell cycle in the cultures. Numbers indicate the percentage of cells in each phase of the cell cycle. **B**, RAW 264.7 cells (2×10^4 cells) were incubated in the absence or presence of RANKL (100 ng/ml) for 4 days. Cells were washed, and total protein was extracted by lysing the cells. Then 50 μ g of extracts was resolved on 10% SDS-PAGE gel, electrotransferred to a nitrocellulose membrane and probed with antibodies to cyclin D1, cyclin D2, cyclin D3, cyclin E, p27, and β -actin as described under "Experimental Procedures." Data is a representative of the three independent experiments.

JNK inhibitor alone. The treatment of cells with RANKL increased the Sub- G_1 fraction to 65%, and the presence of the JNK inhibitor SP600125 reduced this fraction to 26%. Similarly, live/dead assay showed ~45% dead cells with RANKL treatment and ~15% when treated with RANKL along with the JNK inhibitor (Fig. 4D). These results, thus clearly indicate that JNK inhibitor suppresses RANKL-induced apoptosis in Raw 264.7 cells.

RANKL Induces G_0/G_1 Cell Cycle Arrest—RANKL may suppress proliferation through cell cycle arrest. So we next performed cell cycle analysis after treatment of cells with RANKL using PI staining. Data shown in Fig. 5A indicate a decrease in cells in the S and G_2/M phases and a corresponding increase in the G_0/G_1 phase of the cell cycle. These results indicate that RANKL induced G_0/G_1 cell cycle arrest.

When compared with the WT, $I\kappa B\alpha$ -DN RAW 264.7 cells showed a similar decrease of cells in S phase and G_2/M phase

and a corresponding increase of cells in G_0/G_1 phase of the cell cycle (Fig. 5A; lower panel); thus demonstrating the lack of a role for NF- κ B in the anti-proliferative effects of RANKL.

RANKL Down-regulates the Expression of Cyclin D1, D3, and E and Up-regulates p27—Cyclin D and cyclin E together regulate the progression of cells through G_1 to S phase of the cell cycle by regulating the activity of cyclin-dependent kinases (Cdks) (26). Therefore, whether RANKL-induced G_0/G_1 growth arrest was due to down-regulation of cyclin D and cyclin E expression was investigated. As shown in Fig. 5B, treatment with RANKL dose-dependently inhibited the expression of cyclin D1, cyclin D3, and cyclin E proteins. Cyclin D1 was the most sensitive among all the cyclins examined, because down-regulation was noted with as little as 5 ng/ml RANKL. There was a dose-dependent accumulation of the cell cycle inhibitor p27 in cells treated with RANKL. Thus our results are consistent with the thesis that the anti-proliferative effects of RANKL

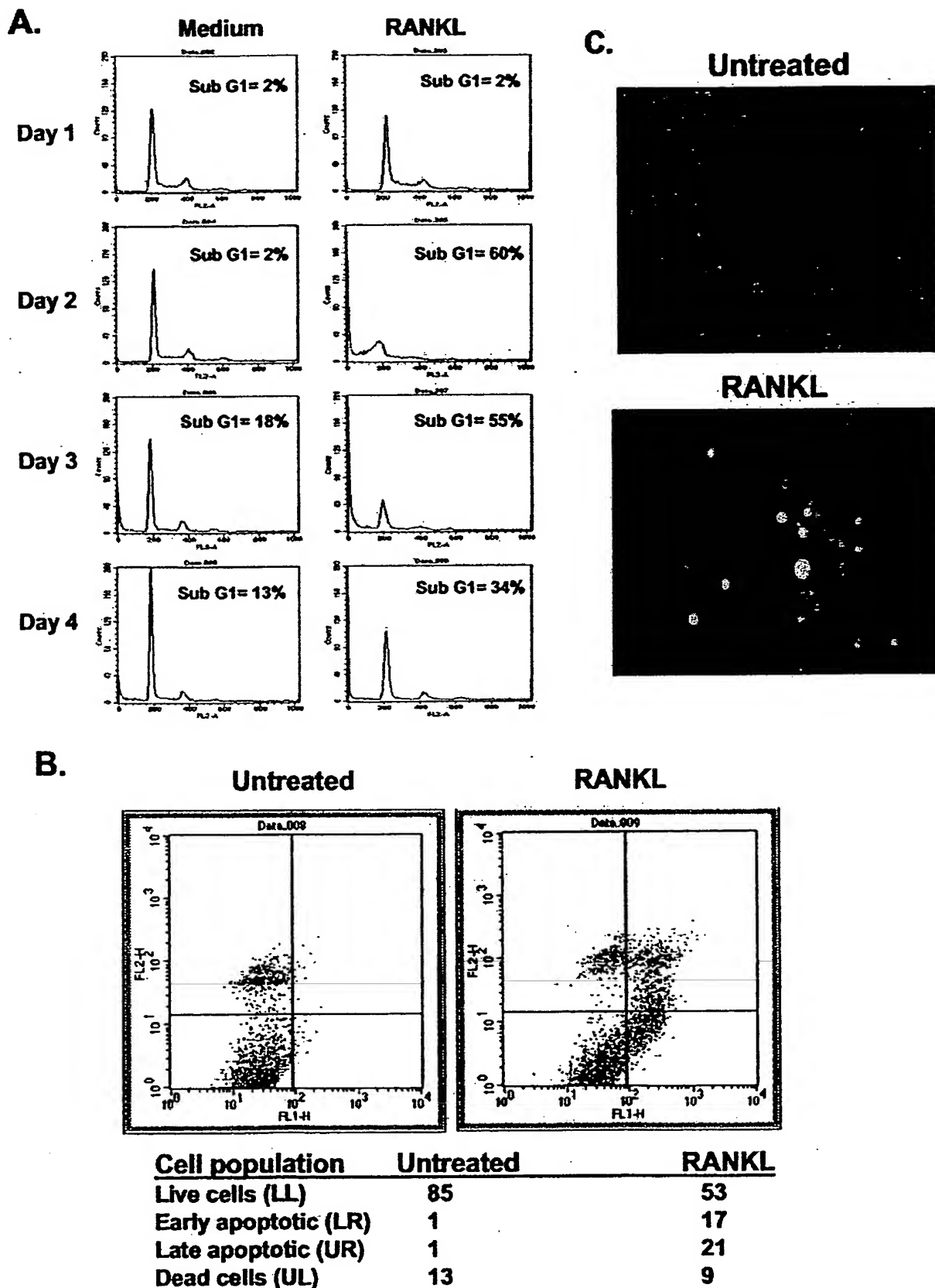


FIG. 6. RANKL induces cell death by apoptosis. A, RAW 264.7 cells (2×10^4 cells) were first cultured in the absence of serum for 2 days and then treated with RANKL (100 ng/ml) for the indicated days. The cells were washed, fixed, stained with propidium iodide, and analyzed for DNA content by flow cytometry as described under "Experimental Procedures." Histograms indicate total cell population in the cultures. Numbers

A.

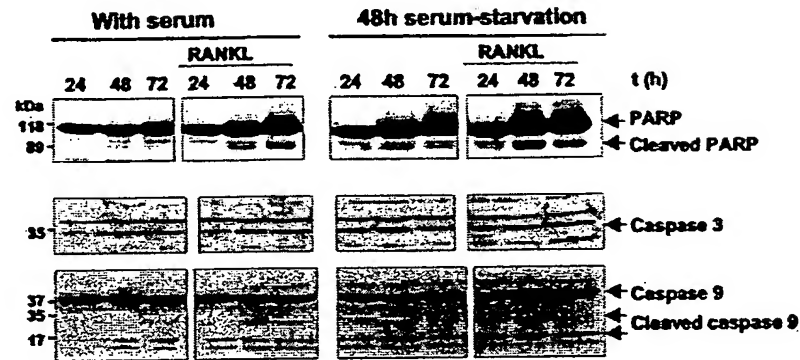
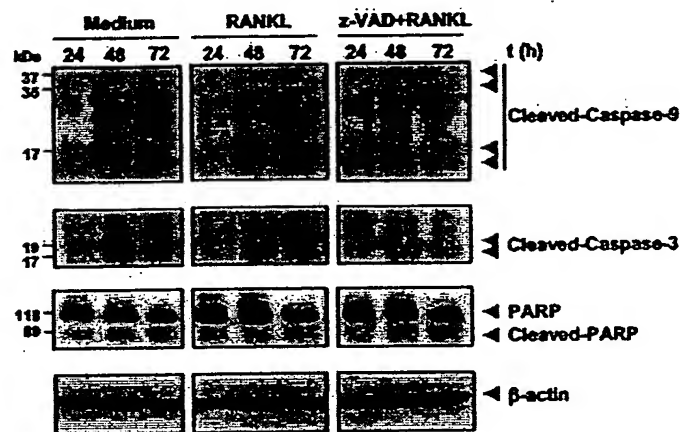


FIG. 7. A, RANKL induces cleavage of PARP, procaspase 3, and procaspase 9. RAW 264.7 cells (2×10^4 cells) were cultured in the presence of serum or in the absence of serum for 2 days and then treated with RANKL (100 ng/ml) for the indicated number of days. Cell lysates were prepared after washing, and lysates were checked for PARP, procaspase 3, and procaspase 9 by Western blotting as described under "Experimental Procedures." B, z-VAD inhibited RANKL-induced cleavage of PARP, procaspase 3, and procaspase 9. RAW 264.7 cells (2×10^4 cells) were cultured in serum-free media for 2 days and then treated with RANKL (100 ng/ml) in the presence or absence of pancaspase inhibitor, z-VAD, for the indicated number of days. Cell lysates were prepared after washing, and lysates were checked for cleaved caspase 9, cleaved caspase 3, and PARP by Western blot analysis as described under "Experimental Procedures." Data are a representative of the three independent experiments.

B.



were due to G_0/G_1 cell cycle growth arrest caused by down-regulation of cyclin D1, D3, and E and up-regulation of p27.

RANKL Treatment Increases the Proportion of Cells with Hypodiploid/Sub- G_1 DNA Content and Induces Apoptosis—Another possible mechanism by which RANKL could mediate its growth inhibitory effects is through induction of cell death. Flow cytometry indicated a rapid increase of cells in sub- G_1 (60%), an indicator of apoptotic cells (27), when cells were serum-starved prior to RANKL treatment (Fig. 6A). We further confirmed the onset of apoptotic cell death by RANKL. Annexin V staining (Fig. 6B) demonstrated a 38% increase in Annexin V-positive cells after exposure of cells to RANKL for 48 h.

A calcein AM-based (live/dead) cytotoxicity assay, which differentiates calcein AM (green)-stained live cells from PI-stained (red) dead cells by fluorescence microscopy, showed that untreated cells primarily took up calcein AM and not PI, whereas RANKL-treated cultures stained with PI (Fig. 6C).

RANKL Induces Cleavage of PARP, Procaspase 3, and Procaspase 9—Because apoptotic cell death is associated with caspase activation, which ultimately results in cleavage of polyadenosine-5'-diphosphate-ribose polymerase (PARP) (28), we next examined the levels of PARP, procaspase 3, and pro-

caspase 9 and their cleavage in RAW cells. PARP cleavage was pronounced in RANKL-treated cells, which were serum-starved prior to RANKL treatment (Fig. 7A, right panels). Untreated cells expressed low levels of procaspase 3. Treatment with RANKL or serum starvation significantly increased the expression of procaspase 3 (Fig. 7A). Cleaved caspase 3 was detected weakly only in cells treated with RANKL following serum starvation (Fig. 7B, middle panels). Similarly, procaspase 9 was also cleaved, because a weak 37-kDa cleavage product of procaspase 9 could be seen only in RANKL-treated cells irrespective of serum starvation (Fig. 7A, lower panel). Pre-treatment of the cells with pancaspase inhibitor, z-VAD, inhibited the cleavage of procaspase 9, procaspase 3, and PARP (Fig. 7B).

RANKL-induced Apoptosis Is Unrelated to Differentiation of Cells—Whether RANKL-induced apoptosis is linked to differentiation of cells, was also investigated. Cells were treated with RANKL for different days and then examined for differentiation by TRAP staining. These results show no significant differentiation of cells up to 3 days after RANKL treatment (Fig. 8). A significant differentiation of cells into osteoclast was noted on days 4 and 5. In contrast, treatment of cells with

indicate the percentage of cells with hypodiploid or sub- G_1 DNA content. B and C, RAW 264.7 cells (2×10^4 cells) were first cultured in the absence of serum for 2 days and then treated with RANKL (100 ng/ml) in the presence of serum for 2 days. Cell death was determined by flow cytometric analysis of Annexin V-stained cells (B) or by calcein AM-based live/dead assay (C) as described under "Experimental Procedures." Numbers indicate the percentage of cells in each quadrant. Data are a representative of the three independent experiments.

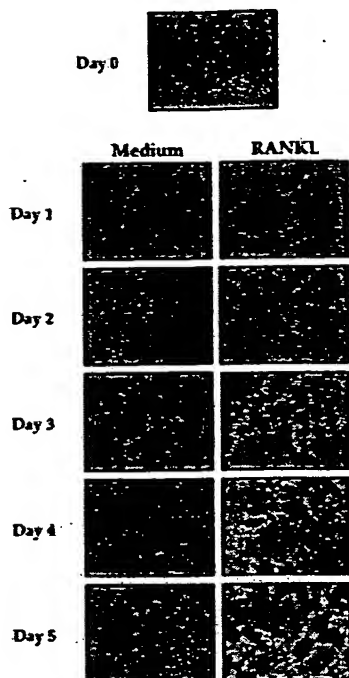


FIG. 8. RANKL induces osteoclast differentiation in RAW 264.7 cells. RAW 264.7 cells (1×10^4 cells) were incubated either alone or in the presence of RANKL (100 ng/ml) for indicated days, stained for TRAP expression and were photographed (original magnification, 100 \times).

RANKL for 2 days induced 60% of cells into sub- G_1 phase (Fig. 6A). Thus these results suggest that RANKL-induced apoptosis is not linked with differentiation.

DISCUSSION

That RANKL regulates osteoclast differentiation and provides a pro-survival signal for certain cell types is well established. Whether RANKL also negatively regulates cell proliferation, was not known before our study. We demonstrated here that treatment of RAW cells with RANKL suppressed the proliferation of these cells. The inhibition was mediated through TRAF6 but was independent of RANKL-induced NF- κ B activation. RANKL-induced JNK activation, however, was found to be essential for growth inhibition. Our results also demonstrated that RANKL induces G_0/G_1 arrest at the same time as levels of cyclinD1, cyclinD3, and cyclin E proteins declined; and CDK inhibitor p27 (Kip) expression increased. RANKL induced apoptosis of cells, as indicated by accumulation of cells in sub- G_0 phase, annexin V staining, calcein AM/ethidium homodimer-1 dye, and an increase in PARP cleavage.

Our results provide clear evidence that, like other members of the TNF superfamily, RANKL can suppress the proliferation and induce apoptosis of cells. Although by itself it is unable to induce apoptosis, RANKL was recently shown to mediate doxorubicin-induced apoptosis in a T lymphoblastic cell line (29). Others, however, have reported that RANKL has an anti-apoptotic effect, up-regulation of Bcl- x_L in dendritic cells (7, 30), or that it has no effect on T and B cells, which also express RANK (30). Therefore, the effects of RANKL appear to be cell type-specific.

The anti-proliferative and apoptotic effects of RANKL were not mediated through NF- κ B activation. RANKL was named based on its ability to activate NF- κ B (2), and NF- κ B activation exhibits both pro-apoptotic and anti-apoptotic effects (31–35). The pro-apoptotic effects of NF- κ B are mediated through ex-

pression of p53 (36, 37), c-Myc (36, 38), FasL (31, 32, 39, 40), TRAIL, and DR4 (41). c-Myc is strongly up-regulated in RAW cells treated with RANKL (42). Whether RANKL can induce other genes is not known. Irrespective of this, RANKL-induced NF- κ B activation was found to be dispensable for the anti-proliferative effects of RANKL. Interestingly, however, its activation is required for the osteoclastic differentiation activity of RANKL (19, 43–47). These results thus distinguish the anti-proliferative effects of RANKL from that of differentiation induction. Our results also suggest that anti-proliferative effects of RANKL were not related to the differentiation effects of the cytokine.

RANKL is also a potent activator of JNK (14, 15). We found that RANKL-induced JNK activation was needed for the anti-proliferative effects of the cytokine. These results are in agreement with recent reports that demonstrate that JNK is required for apoptosis induced by other members of the TNF superfamily, including TNF- α (48), FasL (49), and TRAIL (50, 51). JNK activation is also needed for apoptosis induced by chemotherapeutic agents (52). How JNK mediates apoptosis is not fully understood. For TNF- α -induced apoptosis, the JNK pathway was found to relieve the inhibition imposed by TRAF2-cIAP1 on caspase 8 activation (48).

Our results suggest that anti-proliferative effects of RANKL require the recruitment of TRAF6. TRAF6 has been implicated in signaling via several receptors, including RANK (13, 14), CD40 (53), IL-1R (18, 54, 55), TACI (56), BCMA (57), XEDAR (58, 59), TROY (60), and CD14 (18). Several of these cytokines and lipopolysaccharide have been shown to induce apoptosis (61–64). In addition, TRAF6 deficiency results in osteopetrosis and defective interleukin-1, CD40, and lipopolysaccharide signaling (18). Whether TRAF6 is needed for anti-proliferative effects and induction of apoptosis by these ligands is not known, but a recent report by Lomaga *et al.* (65) indicates an essential role for TRAF6 in apoptosis induction in the developing CNS. They used a TRAF6 gene knockout mouse model to demonstrate this.

Our results indicate that the anti-proliferative effects of RANKL are due to G_0/G_1 cell cycle arrest. We also demonstrated that G_0/G_1 arrest accompanied with down-regulation of cyclin D1, D3, and cyclin E and up-regulation of the CDK inhibitor p27 (Kip). Our results are in agreement with a previous report that showed induction of p27 by RANKL in bone marrow macrophages (66). TNF, FasL, and TRAIL have been shown to induce cell growth arrest but through different mechanisms (67–69). Interestingly TNF induced G_1 arrest in synchrony with reductions in the levels of cyclin D1 and increases in p27^{Kip1} expression (67). Whether other members of the TNF superfamily can induce cell cycle arrest, inhibit cyclin expression, or induce the expression of cell cycle inhibitors is not known.

We also demonstrated that RANKL induced apoptosis as indicated by DNA analysis, annexin V staining, calcein AM/EtBr assay, and PARP cleavage. It is known that TNF, Fas, and TRAIL can induce apoptosis in a variety of cells (70–72). Although TNF can activate NF- κ B in all cells, Fas and TRAIL activate NF- κ B only in a few selected cells, irrespective of receptor expression. In contrast, RANKL appears to activate NF- κ B in all cells that display the RANK receptor. Whether RANKL can suppress proliferation of all cells that express receptors is not clear at present.

The activation of apoptosis by TNF, Fas, and TRAIL requires the recruitment of FADD and FLICE proteins to the receptor. Whether RANK can recruit these proteins is not understood. APRIL, VEGI, and CD40L have also been shown to suppress cell growth and induce apoptosis in certain cells (73–76), but

whether it is mediated through FADD and FLICE recruitment is again unclear. Like RANKL, receptors for APRIL and CD40L have been shown to lack the death domain (for references see Ref. 72).

Our results implicate TRAF6 in suppression of proliferation and induction of apoptosis. Overall our results demonstrate that, like other members of the TNF superfamily, RANKL can also suppress cell proliferation and induce apoptosis through a mechanism that is not regulated by NF- κ B activation but does require the recruitment of TRAF6.

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